A “dilute and shoot” method for measuring norpropoxyphene in human urine using liquid chromatography–tandem mass spectrometry distinguishes two different metabolites of propoxyphene, norpropoxyphene (m/z 326) and a dehydrated rearrangement product (m/z 308). The metabolite formed from the rearrangement and dehydration of norpropoxyphene is excreted in human urine and also may be formed from the chemical degradation of norpropoxyphene. Previously, these two metabolites were indistinguishable by gas chromatography–mass spectrometry methods that use an alkaline extraction that converts norpropoxyphene into its rearrangement product. The degradation of norpropoxyphene presents a challenge for its analytical quantitation, and methods for circumventing these issues are presented.

Introduction

Propoxyphene is a prescribed narcotic analgesic, structurally similar to methadone. Measurement of norpropoxyphene in urine is commonly performed to confirm propoxyphene ingestion (1), as it is a major excreted metabolite. Traditionally, immunoassay methods have been used to screen for the presence of propoxyphene, with positive screens confirmed using gas chromatography–mass spectrometry (GC–MS) to detect norpropoxyphene.

According to Baselt (2), “There are a number of specific gas chromatographic procedures for the assay of propoxyphene and norpropoxyphene in the literature … In the latter procedure norpropoxyphene is treated with strong base before the final extraction, converting it into an internal amide in order to improve its chromatographic properties.” Typical GC–MS procedures do not detect norpropoxyphene, but instead detect its alkaline rearrangement product. “Dilute and shoot” methods employing liquid chromatography–tandem mass spectrometry (LC–MS–MS), on the other hand, do not involve alkaline extraction and are capable of distinguishing between two different metabolites of propoxyphene, norpropoxyphene (m/z 326) and a dehydrated rearrangement product (m/z 308), which have previously been indiscernible, or lumped together, by GC–MS methods. LC–MS–MS measurements presented here demonstrate that the alkaline extraction step of GC–MS methods converts norpropoxyphene into a dehydrated rearrangement product.

Previous studies based on isotope labeling have inferred the existence of cyclic norpropoxyphene as a circulating metabolite in dog plasma (3). Although this metabolite is indiscernible from norpropoxyphene with traditional GC–MS methods, it is clearly distinguishable from norpropoxyphene when using LC–MS–MS methods. The product resulting from the rearrangement and dehydration of norpropoxyphene is not only an excreted metabolite in human urine but may also be formed by chemical degradation of norpropoxyphene. Its degradation presents a challenge to quantitation of norpropoxyphene, and in addition, because of the presence of varying ratios of the two metabolites in human urine, different quantitative values may be obtained for norpropoxyphene, depending on the choice of analytical methods and extraction procedures.

Methods

We perform quantitative LC–MS to identify and confirm the presence of norpropoxyphene in patient urine samples. During the development of our assay, we discovered the presence of two propoxyphene-related compounds in certified standards and patient urine samples by LC–MS–MS. We validated the observation of two metabolites based on differences in their chromatographic retention times and full scan mass spectra. All experiments were performed on an identical instrumental platform and setup.

An Agilent 1200 series binary pump SL LC system, well plate
samples were collected using electrospray ionization with the MS running in positive mode. The optimized instrumental parameters were as follows: gas temperature, 350°C; drying gas, 12 L/min; nebulizer gas (nitrogen), 35 psi (~24,100 Pa); capillary voltage, 3000 V; fragmentor voltage, 60. Multiple reaction monitoring (MRM) mode was used for quantitation and product ion scan (PIS) mode was used to acquire full scan MS–MS spectra. In PIS mode MS–MS spectra were collected with collision energy set to 5, 10, 20, and 30 volts. Product ion spectra were collected in the range of m/z 50 to 300. Scan time was set to 500 ms.

In full scan mode, two transitions are used to analyze a single compound. A quantitative transition is used to calculate concentration based on the quantifier ion and a qualitative transition is used to ensure accurate identification of the target compound based on the ratio of the qualifier ion to the quantifier ion. The following quantitative transitions were used:

- 326 → 252 with collision energy set to 5 volts for norpropoxyphene
- 308 → 143 with collision energy set to 20 volts for dehydrated rearrangement product
- 326 → 308 with collision energy set to 10 volts for rearrangement product
- 313 → 147 with collision energy set to 20 volts for norpropoxyphene-d5 (norpropoxyphene-d5 is the dehydrated rearrangement product of deuterated norpropoxyphene). The following qualitative transitions were used:
  - 326 → 44 with collision energy set to 10 volts for norpropoxyphene and 308 → 143 with collision energy set to 20 volts for dehydrated rearrangement product. No qualitative transition was used for the rearrangement product. Dwell times were set to 50 ms.

Quantitative analysis is performed using Agilent Mass Hunter Quantitative Analysis software. A four point calibration curve is created by using a linear fit and forcing the origin. Accepted accuracy for calibrators is ±20% of the target value and the coefficient of determination (R^2) is required to be greater than or equal to 0.99 as verification of linearity and goodness-of-fit. The measured upper limit of linearity for the norpropoxyphene assay is 100,000 ng/mL. The assay has a day-to-day coefficient of variation of 10%. This precision analysis is based on 20 measurements of 2 prepared quality control samples, 1 at 100 ng/mL, and 1 at 1000 ng/mL. The measurements were performed over the course of 1 month.

HPLC-grade water, acetonitrile, methanol, and formic acid were obtained from VWR (Westchester, PA). Norpropoxyphene with a concentration of 100 µg/mL (calibrator 1) and deuterated norpropoxyphene-d7 standard with a concentration of 100 µg/mL in methanol were obtained from Cerilliant (Round Rock, TX), and diluted to 1000 ng/mL in synthetic urine (Microgenics, Fremont, CA) or methanol. Norpropoxyphene in dried powder form was obtained from Sigma-Aldrich (St. Louis, MO). The powder was dissolved to 1000 ng/mL in synthetic urine (calibrator 2). Standards were stored at −20°C. Norpropoxyphene calibrator 2 was treated with 5 N NaOH to produce its alkaline rearrangement product.

ASPIRE Independent Review Board of La Mesa, California approved the use of patient samples for this study. Patient samples were supplied by pain management patients and received by Millennium Laboratories from physicians treating the patients for pain. Patient samples shipped to us were stored a 4°C upon their arrival.

**Results**

LC–MS–MS analysis of norpropoxyphene standards reveals two forms of norpropoxyphene exist in solution. One form is parent norpropoxyphene with molecular ion m/z 326. The other is a product formed by the rearrangement and dehydration of norpropoxyphene with molecular ion m/z 308. Chromatographic runs are shown for stan-
standards supplied from separate vendors in Figure 1 showing the presence of norpropoxyphene and its dehydrated rearrangement product in both standards. Retention times, which are labeled on the x-axis, are different for the two forms of the analyte. At mobile phase conditions of 65% A and 35% B, norpropoxyphene elutes at 3.4 min and its dehydrated rearrangement product elutes at 3.5 min. We were able to achieve slightly better chromatographic separation of 0.3 min between m/z 308 and m/z 326 with mobile phase conditions of 80% A and 20% B; however, this increased total runtime to 15 min, and peak shape was compromised. Norpropoxyphene and its dehydrated rearrangement product are not isobaric; therefore, the shorter, more robust run was used to quantitate the analytes. Figure 1 shows the extracted ion chromatograms of m/z 308 and m/z 326 for calibrators 1 and 2 acquired from their full scan mass spectra. The chromatographic peak at 3.5 min shows a single ion at m/z 308; however, the chromatographic peak at 3.4 min shows two ions at m/z 326 and m/z 252. The two ions observed in the peak at 3.4 min are due to norpropoxyphene forming an unstable molecular ion and partially dissociating in the ion source of the MS to form m/z 252. When we increased the fragmentor voltage from 60 to 120 volts for injections of calibrator 2, we observed approximately a twofold increase in the ratio of m/z 252 to m/z 326. Under the same conditions, the dehydrated rearrangement product (m/z 308) remained intact.

The chromatographic peak areas of calibrators 1 and 2 shown in Figure 1 may be used to estimate the purity of each calibrator based on the assumption that norpropoxyphene and its dehydrated rearrangement product have similar ionization efficiencies and that together they comprise 100% of each calibrator. The purities of calibrators 1 and 2 are estimated according to the peak areas of the extracted ion chromatograms in Figure 1. In calibrator 1, m/z 308 has a peak area of approximately 506,000,000 counts, and m/z 326 has a peak area of approximately 16,000,000 counts. In calibrator 2, m/z 308 has a peak area of approximately 28,000,000 counts, and m/z 326 has a peak area of approximately 430,000,000 counts. According to previously listed assumptions, calibrator 1 is approximately 97% dehydrated rearrangement product (m/z 308) and 3% norpropoxyphene. Calibrator 2 is approximately 93% norpropoxyphene (m/z 326) and 7% dehydrated rearrangement product. By ignoring the in-source fragmentation of norpropoxyphene, the purity of calibrator 2 is underestimated; however, the estimate of 93% gives less than a 10% error in the calculated concentrations, which is acceptable.

Table 1 shows the effect of storage on norpropoxyphene calibrator 2 over a course of 21 days, stored at 4°C in four solvents: methanol, synthetic urine, acetonitrile, and H₂O with 10% ethanol added. The solutions were prepared from norpropoxyphene calibrator 2, which was prepared from solid norpropoxyphene stored at −20°C. The calibrator was diluted to a concentration of 1000 ng/mL in the four solvent types and aliquoted into 16 separate vials. Concentrations for norpropoxyphene (m/z 326) were based on a calibration curve produced with calibrator 2, which was prepared from solid norpropoxyphene stored at −20°C until the day of measurement and then dissolved in synthetic urine. Concentrations for the dehydrated rearrangement product were based on a calibration curve produced with calibrator 1, which was prepared from an ampoule of standard in methanol which was stored unopened at −20°C until the day of measurement and then diluted in synthetic urine. The 16 vials were measured on the day of preparation, then stored for 3 weeks at 4°C and re-measured using freshly prepared calibrators as described. The average calculated concentration for m/z 326 and m/z 308 in each solution is shown in Table 1 along with the standard deviation in the calculated concentrations for each time point and solvent. The calculated concentrations are up to 7% lower than reported since they are based on calibrators that have estimated purities of 93–97%. The values in Table 1 show that the concentration of norpropoxyphene decreased by the least amount when it was stored in methanol, and increased by the largest amount when it was stored in synthetic urine. This coincides with the observation that amount of dehydrated rearrangement product also increased by the least amount when it was stored in methanol and by the least amount when it was stored in synthetic urine. This may explain why norpropoxyphene standards supplied in methanol contain approximately 97% dehydrated rearrangement product and only 3% norpropoxyphene. These results support the chemical degradation of norpropoxyphene into a dehydrated rearrangement product. Previous reports conclude that at somewhere between 3 and 6 months in the freezer at −20°C, norpropoxyphene in urine degrades to less than 50% recovery, in agreement with our find-

<table>
<thead>
<tr>
<th>Table 1. Stability of Norpropoxyphene Over Three Weeks at 4°C*</th>
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<td></td>
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<tr>
<td>Methanol</td>
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<td>1 day</td>
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<td>21 days</td>
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<tr>
<td>Synthetic urine</td>
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<td>1 day</td>
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<td>21 days</td>
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<tr>
<td>Acetonitrile</td>
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<tr>
<td>1 days</td>
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<tr>
<td>21 days</td>
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<tr>
<td>H₂O (10% ethanol)</td>
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<tr>
<td>1 day</td>
</tr>
<tr>
<td>21 days</td>
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</table>

* The concentrations of norpropoxyphene and dehydrated rearrangement product are based on calibrators that are approximately 93–97% pure; therefore, the accuracy of reported concentrations is limited. Actual concentrations of patient samples are estimated to be up to 7% lower than those reported.
The degradation of norpropoxyphene is observed to be much slower for the solid form of norpropoxyphene, as calibrators prepared from the solid form of norpropoxyphene stored at –20°C for up to 6 months show an unobservable increase in the ratio of dehydrated rearrangement product to norpropoxyphene. Therefore, to obtain the most accurate results it is necessary to routinely prepare fresh calibrators from solid norpropoxyphene stored at –20°C.

To determine the effect of typical GC–MS preprocessing procedures both calibrators 1 and 2 were subjected to an alkaline extraction using 5 N NaOH (6–8). Analysis by LC–MS–MS showed that after alkaline extraction the concentration of norpropoxyphene decreased to a negligible amount. The amount of rearrangement product increased. The observed MS–MS spectra of norpropoxyphene, its rearrangement product, and its dehydrated rearrangement product are shown in Figure 2. The MS–MS spectra of the rearrangement product and the dehydrated rearrangement product shown in Figure 2 were measured using samples of calibrator 2 subjected to an alkaline extraction. Because we only observed the rearrangement product in alkaline extracted samples with drastically altered pH, we were unable to determine the retention time of the rearrangement product relative to norpropoxyphene, which was unobservable in samples subjected to alkaline extraction. The MS–MS spectra of alkaline extracted samples were compared to both calibrators 1 and 2. The MS–MS spectra of m/z 308 in alkaline extracted samples of calibrator 2 matched the MS–MS spectrum of m/z 308 in calibrator 1 (not subjected to alkaline extraction), leading to the conclusion that typical GC–MS procedures involving alkaline extraction convert norpropoxyphene into its dehydrated rearrangement product. The main fragmentation pathways observed in the MS–MS spectra coincide with the proposed structures presented in Figure 2 and are illustrated by chemical structures and fragmentation lines.

A number of control and patient samples were tested to determine whether norpropoxyphene or its dehydrated rearrangement product were present. Concentrations for norpropoxyphene and its dehydrated rearrangement product may actually be up to 7% lower than reported in Table II, based on the purity estimates of calibrators 1 and 2 described previously. Norpropoxyphene and its dehydrated rearrangement product were quantitated in dilute urine samples of 15 human patients and 2 quality control samples (BIORAD and UTAK), with supplier assayed values of 1000 and 250 ng/mL, respectively. The results of these analyses are presented in Table II and show that norpropoxyphene and its dehydrated rearrangement product are present in patient urine. The amount of dehydrated rearrangement product present ranges from 16 to 81% of the total amount of norpropoxyphene excreted. These findings support the previously proposed existence of a metabolite formed from the cyclization of norpropoxyphene (3).

### Discussion
Norpropoxyphene is a part of our confirmatory drug analysis using LC–MS–MS. A “dilute and shoot” procedure for quantitating norpropoxyphene in human urine with LC–MS dis-

<table>
<thead>
<tr>
<th>Patient Urines*</th>
<th>Dehydrated Rearrangement Product Concentrations in Patient Urines*</th>
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<tbody>
<tr>
<td></td>
<td>Norpropoxyphene Concentration (ng/mL)</td>
</tr>
<tr>
<td>BioRad QC</td>
<td>1031</td>
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<tr>
<td>Utak QC</td>
<td>320</td>
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<tr>
<td>Pt 1</td>
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<tr>
<td>Pt 2</td>
<td>270</td>
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<td>Pt 3</td>
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<td>Pt 15</td>
<td>31006</td>
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<td>Pt 16</td>
<td>3995</td>
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</table>

*The concentrations of norpropoxyphene and dehydrated rearrangement product are based on calibrators that are approximately 93–97% pure; therefore, the accuracy of reported concentrations is limited. Actual concentrations of patient samples are estimated to be up to 7% lower than those reported.
tinguishes two similar metabolites of propoxyphene, nor-
propoxyphene and a dehydrated rearrangement product. Both
forms were found to be excreted in the urines of patients pre-
scribed propoxyphene. The two mass ions have not been rou-
tinely observed by GC–MS because the preanalytical procedure
which uses strong alkali converts norpropoxyphene to a rear-
rangement product and its dehydrate. One of our colleagues
(Robert S. White, personal communication) sent us a GC–MS
spectrum of the common method for analyzing nor-
propoxyphene. In this analysis m/z 307 was observed as the
major ion using electron impact ionization. This mass analyte
is in agreement with the dehydrated rearrangement product
that we observe in norpropoxyphene standards and additionally
identify as a product of alkaline extraction of norpropoxyphene.
Our findings also suggest the dehydrated rearrangement prod-
uct is formed by chemical degradation of norpropoxyphene.

For those doing analytical work, it is demonstrated that
GC–MS and LC–MS–MS procedures may give different quan-
titative answers as a result of differences in specimen prepara-
tion. This suggests that “dilute and shoot” methods based on
LC–MS–MS technology will likely identify other potentially
novel drug metabolites. The observation that the expected nor-
propoxyphene metabolite is accompanied by significant
amounts of the dehydrated rearrangement product in patient
urine and that both can be quantified by LC–MS–MS raises the
question of how to quantify norpropoxyphene. GC–MS meth-
ods using alkaline extraction lump the metabolites together.
LC–MS–MS “dilute and shoot” methods differentiate the two
metabolites.

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   propoxyphene and nordextropropoxyphene in biological fluids.
   Gas chromatographic quantitation of dextropropoxyphene and
   nordextropropoxyphene in urine after solid phase extraction.

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